CAROTENE BIOSYNTHESIS IN FUNGI

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ABSTRACT

Studies on *Neurospora crassa* and mutants of *Phycomyces blakesleeanus* have led to a reappraisal of a number of important aspects of carotene biosynthesis. Results obtained from structural investigations, from inhibitor studies and from the use of labelled substrates with carotenogenic extracts of fungal mutants are discussed in the contexts of the stereochemistry, isomerization and dehydrogenation of phytoene, the pathway of carotene cyclization and the feedback control of the biosynthetic pathway.

INTRODUCTION

Studies on a number of fungi, including *Neurospora crassa, Blakeslea trispora, Mucor hiemalis* and, particularly, *Phycomyces blakesleeanus*, have figured prominently in the literature of carotenoid biosynthesis over the last twenty years. That they have done so is largely attributable to certain advantages that fungi have over many other organisms as experimental systems for studies of carotenogenesis. Their carotenoid content is relatively simple, so that investigations of their hydrocarbon carotenes are, in general, not complicated by the presence of the oxygenated xanthophylls, which are more characteristic of higher plants, algae and bacteria. The ease with which they could be cultured, and the nutrients and other culture conditions varied, meant that they were ideal organisms for many of the early and important studies which laid the foundations of our present knowledge of carotenoid biosynthesis.

It is appropriate at this time to recall the contributions made to this field by the late Professor Grob who, with Schopfer[1], first demonstrated the stimulation by acetate of β-carotene (β,β-carotene) formation in *P. blakesleeanus* and who, using [1-^{14}C]- and [2-^{14}C]acetate, showed that β-carotene is formed by *M. hiemalis* from repeating isoprene units[2–4]. Goodwin, Mackinney and Chichester and their co-workers[5–8] observed and explained the stimulatory effects of leucine and related compounds on β-carotene formation in *P. blakesleeanus*, and this same organism was among the experimental systems they used for their demonstrations that mevalonic acid (MVA) is as effective a precursor of carotenes as it is of sterols[7,8]; Grob again used *M. hiemalis*[9], while Krzeminski and Quackenbush used *N. crassa*[10], to demonstrate the conversion of MVA into carotenes. Many other intermediates of squalene and sterol formation were shown to be equally efficient as carotene precursors. Chichester and his collaborators,

using a cell-free system from *P. blakesleeanus*, obtained indirect evidence for the participation of isopentenyl and dimethylallyl pyrophosphates in carotene synthesis[11] and, by direct incorporation experiments in essentially the same type of system, demonstrated that farnesyl pyrophosphate (FPP)[12] and geranylgeranyl pyrophosphate (GGPP)[13] are converted into carotenes. Studies on the hydrocarbons of *N. crassa*[14] were partly instrumental in establishing phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene) as the first conjugated C_{40}-intermediate in carotenoid biosynthesis. The possible biosynthetic interrelationships between this colourless polyene and the coloured acyclic and cyclic carotenes were explored by studying the effect of diphenylamine (DPA) on *P. blakesleeanus*[15, 16], and the observation of the formation of β-zeacarotene (7′,8′-dihydro-β,ψ-carotene) under these conditions[17, 18] is an important feature of any discussion of carotene cyclization. Whatever the precise identity of the immediate acyclic precursor of the cyclic carotenes, an attractive general mechanism for the cyclization reaction has been obtained by Goodwin and Williams, who studied the incorporation of stereospecifically labelled MVA into phytoene and β-carotene by *P. blakesleeanus*[19].

The experiments reported here, many of them of a preliminary nature, represent an attempt to investigate, largely in one organism, some of those aspects of carotene biosynthesis which have now held the attention of workers for a number of years. These include the formation of the C_{40}-polyene, phytoene, its stereochemistry and its role as a precursor of the more unsaturated carotenes, the pathway of carotene cyclization involved in the formation of β-carotene, and the control of carotene biosynthesis. The experimental approaches have been varied and have included physical organic structural analyses, the use of inhibitors of carotene biosynthesis and studies both of the incorporation of low molecular weight precursors into carotenes and of the metabolism of labelled carotenes by crude enzyme systems.

MUTANTS OF *PHYCOMYCES BLAKESLEEANUS*

These investigations have been carried out very largely on three mutants of *P. blakesleeanus*. These mutants, obtained by *N*-methyl-*N*-nitroso-*N*′-nitroguanidine treatment of vegetative spores of *P. blakesleeanus* (NRRL 1555), were kindly provided by Drs M. Delbrück and K. Bergman; their carotenes have been described by Meissner and Delbrück[20]. According to the recommendations of the Caltech Group on Phycomyces Genetics Nomenclature[21], mutants which differ from the wild type in their ability to form β-carotene are designated '*car*', while those strains which differ from the wild type in their phototropic response, but not in their morphology, are designated '*mad*'. Thus the yellow mutant used in these studies (strain C115 and formerly called Ph 107), which accumulates exclusively β-carotene at 5 times the concentration in the wild type, yet differs from the latter in its reaction to light, is designated *mad-107*(−)[22]. The other two mutants that have been used differ from the wild type in their ability to form β-carotene. The white mutant (strain C5 and formerly called alb 10) is designated *car-10*(−); it accumulates phytoene at a concentration approximately twice

that of the β-carotene in the wild type. The *carR21*(−) mutant (strain C9 and formerly called rl) forms a number of carotenes, among which lycopene (ψ,ψ-carotene) predominates at a concentration some 5 times that of the wild type β-carotene; its red colour leads to the designation '*R*' for the locus affected by this particular mutation. The details of some of our analyses of the carotenes of the three mutants are shown in *Table 1*.

Table 1. Concentrations of carotenes in mutants of *Phycomyces blakesleeanus* grown in shake culture in light and in darkness

Carotene	Carotene concentration* (μg/g dry weight)					
	carR21(−)		*mad-107*(−)		*car-10*(−)	
	Light	Dark	Light	Dark	Light	Dark
Phytoene	772	515	—†	—	740	500
Phytofluene	63	109	—	—	tr‡	tr
ζ-Carotene	45	56	—	—	—	—
Neurosporene	tr	tr	—	—	—	—
Lycopene	1546	1206	—	—	—	—
β-Zeacarotene	tr	tr	—	—	—	—
γ-Carotene	14	3	—	—	—	—
β-Carotene	tr	tr	1420	1210	—	—
Total carotenes	2440	1889	1420	1210	740	500

* These carotene concentrations are qualitatively the same but quantitatively higher than either those quoted for sporangiophores[20] or, in the case of the *carR21*(−) mutant, those quoted for mycelia grown on a medium which, in contrast to that used here, did not contain leucine and asparagine[13]

† Not detected

‡ Trace, less than 1 μg/g

These three mutants present an attractive combination of experimental systems for the study of a number of features of carotene biosynthesis. While the *mad-107*(−) mutant is clearly capable of an extremely efficient cyclization of the appropriate acyclic precursor to form β-carotene, the *carR21*(−) strain represents an experimental system in which studies of the dehydrogenation of phytoene to lycopene should be relatively uncomplicated by cyclization reactions. The white *car-10*(−) mutant, in which the formation of one compound, phytoene, represents its total capacity for C_{40}-synthesis, should be an ideal model system for comparatively simple studies of the control of carotene and sterol formation.

CELL-FREE SYSTEMS FROM *PHYCOMYCES BLAKESLEEANUS*

In order to carry out detailed investigations using labelled substrates, the development of carotenogenically active extracts is essential. Previous attempts to prepare such systems from *P. blakesleeanus* have met with varying success. Yokoyama *et al.*[11] homogenized mycelial mats of the fungus to yield a crude enzyme system with which, after it had been dialysed, they were able to demonstrate an 8.2 per cent conversion of the active isomer of DL-[2-^{14}C]MVA into β-carotene in 24 hours. The same method has also yielded a cell-free system from the *carR21*(−) mutant[13] but this, over the

same incubation period, only showed 5.8 per cent and 1.7 per cent incorporations of [2-^{14}C]MVA into the total unsaponifiable and total carotene fractions respectively, although some 19 per cent of the radioactivity from [^{14}C]GGPP was incorporated into the carotenes. Other methods of disrupting and extracting *P. blakesleeanus* have also been used. Crude enzyme systems prepared from the *car-10*(−) mutant by the methods of Goulston and Mercer[23] (grinding lyophilized mycelia in buffer with glass beads) and of Mercer and Johnson[24] (crushing mycelia under liquid nitrogen) gave, in our studies, some 10–15 per cent incorporations of [2-^{14}C]MVA into the unsaponifiable fraction. The cell-free preparations used in the present series of studies are simply buffer extracts of sieved lyophilized mycelia from shake cultures of the *P. blakesleeanus* mutants. When incubated for 3 hours under the appropriate conditions, they will consistently incorporate some 40–50 per cent of the activity of [2-^{14}C]MVA into the unsaponifiable fraction; the highest incorporation recorded[25], for an extract of the *car-10*(−) mutant, is 75 per cent.

FORMATION OF PHYTOENE, SQUALENE AND STEROL

Incubations of [2-^{14}C]MVA with the enzyme system from the *car-10*(−) mutant of *P. blakesleeanus* were carried out for 3 hours at 24°C in the dark: all incubations were supplemented with ATP, Mg^{2+}, Mn^{2+} and glutathione (GSH; 10, 4, 6 and 20 μmoles/ml respectively). Incorporations of radioactivity into the unsaponifiable components showed the now expected response to varying the incubation conditions and the cofactors[26]. In the presence of NADPH (1 μmole/ml) and air, some 16 per cent of the available radioactivity from the [2-^{14}C]MVA was incorporated into the sterol fraction, but this figure was much reduced under anaerobic conditions, when squalene was formed rather than sterol. A clear requirement for NADPH could be demonstrated for the formation of squalene under anaerobic conditions; while the incorporations of available radioactivity into phytoene and squalene in the presence of NADPH were 6 and 14 per cent respectively, the absence of the cofactor or its replacement by any other of the nicotinamide cofactors resulted in zero incorporation into squalene, with a slight increase in the radioactivity appearing in the phytoene.

Another compound became radioactive in the course of the incubations with [2-^{14}C]MVA as long as NADPH was present. This is an unidentified hydrocarbon, less polar than squalene, which might be either a di- or tetrahydrosqualene or a product resulting from a proton-initiated cyclization of squalene. While the incorporation of radioactivity into this compound is virtually undetectable in the presence of oxygen, it accounts for some 2 per cent of the available radioactivity in anaerobic incubations. Evidence is also available for the formation of this compound from [^{14}C]squalene. Thus while the inhibition of sterol formation by the absence of NADPH results in the rechannelling of isoprenoid biosynthesis into phytoene, its inhibition by the absence of oxygen results in some formation of an alternative product of squalene metabolism.

Since the *car-10*(−) mutant of *P. blakesleeanus* normally accumulates such a high concentration of phytoene, it was considered worthwhile to

investigate whether any lycopersene (7,8,11,12,15,7′,8′,11′,12′,15′-decahydro-ψ,ψ-carotene) was formed by the crude enzyme system. As a routine control in many of these experiments, carrier lycopersene was added together with squalene, phytoene and ergosterol. In no case was any radioactivity recovered in the reisolated lycopersene. Although lycopersene is not generally considered as being an intermediate in carotenoid biosynthesis[27], its formation from GGPP in the presence of NADPH by a system capable of forming squalene might be anticipated if the squalene synthetase enzyme were not absolutely specific for FPP. Indeed, such a situation might be the explanation of the observation that an enzyme system from *N. crassa* is capable of forming lycopersene from GGPP[28].

STEREOCHEMISTRY OF PHYTOENE

The stereochemistry of the central triene chromophore of phytoene is of crucial importance to carotenoid biosynthesis. Studies on samples of phytoene isolated from tomatoes, from carrot oil and from a mutant of *Chlorella vulgaris* showed that all have a *cis* configuration at the central 15-double bond[29, 30]. The 15-*cis* phytoene isolated from carrot oil and tomato paste is accompanied by traces of another isomer, apparently all-*trans*, which, it is suggested[29], may be formed by isomerization during processing. While the formation of 15-*cis* phytoene is consistent with the $^{3}H/^{14}C$ ratios of phytoene formed by higher plant systems from (5*R*)-[2-^{14}C-5-$^{3}H_1$]- and [2-^{14}C-5,5-$^{3}H_2$]MVA[31, 32], it must undergo a subsequent isomerization to form the normal all-*trans* carotenoids. This isomerization could take place at the phytoene level or at a later stage in the dehydrogenation sequence.

Recent studies by Aung Than *et al.*[33] have compared the stereochemistry of samples of phytoene isolated from a number of natural sources, including some fungal samples (*Table 2*). Phytoene from *N. crassa*, from DPA-inhibited

Table 2. The levels of 15-*cis* and all-*trans* phytoene in some fungi and other natural sources[33]

Source of phytoene	Wet or dry wt. of original material	Total phytoene (mg)	Percentages of isomeric phytoenes	
			15-*cis*	all-*trans*
Phycomyces blakesleeanus:				
Wild type (100 μM DPA)	206 g (wet)	5.8	98.7	1.3
car-10(−) mutant	28.6 g (dry)	9.8	96.8	3.2
Neurospora crassa	400 g (wet)	16.5	98.0	2.0
Tomato fruit	3.6 kg (wet)	12.0	97.2	2.8
Carrot oil	4.8 g	6.6	99.8	0.2
Rhodospirillum rubrum (65 μM DPA)	24.0 g (dry)	17.8	85.1	14.9

cultures of wild type *P. blakesleeanus* and from the phytoene-accumulating *car-10*(−) mutant have a number of features in common. All the fungal samples are predominantly of the 15-*cis* configuration, but all contain between 1 and 4 per cent of what is apparently the all-*trans* isomer as a naturally

occurring component. Comparisons of the u.v., i.r. and 100 and 220 MHz n.m.r. spectra with those of stereochemically unambiguous triene models, synthesized by Khatoon *et al.*[34], have enabled a *trans, cis, trans* configuration to be assigned to the central triene chromophore of the main component of fungal phytoene.

DIPHENYLAMINE-INDUCED PHYTOENE ACCUMULATION

The recognition of the natural occurrence of the two isomeric phytoenes has prompted a reinvestigation of the effects on polyene levels of culturing *P. blakesleeanus* in the presence of DPA and then removing the inhibitor and resuspending the mycelium in buffer. Previous reports that the formation of the normal unsaturated carotenes on resuspension is not accompanied by a corresponding decrease in the level of accumulated phytoene could be explained on the basis of the all-*trans* rather than the 15-*cis* isomer of phytoene being the actual substrate for dehydrogenation reactions. A total inhibition of lycopene formation and an accumulation of phytoene result from culturing the *carR21*(−) mutant of *P. blakesleeanus* in the presence of 50 μM DPA[35]. Resuspension of the mycelium in buffer after the removal of the inhibitor results in a change in colour from a very pale yellow to bright orange over a period of 2 hours, after which the colour fades. Repeated attempts, using different conditions and resuspension buffers of different composition, have failed to regenerate the characteristic red colour of this lycopene-accumulating mutant.

Table 3. Changes in carotene content of mycelia of the *carR21*(−) mutant of *Phycomyces blakesleeanus*, grown in the presence of 50 μM DPA, on harvesting and resuspending in phosphate buffer (0.2M, pH 7.0)

Carotene	Carotene concentration (μg/g dry weight)					
	At harvest	Time after resuspension (hours)				
		0	0.5	1	2	3
15-*Cis* phytoene	560	554	464	475	426	341
All-*trans* phytoene	53	34	73	161	69	51
Phytofluene	22	26	40	29	27	18
ζ-Carotene	tr*	5	8	7	8	2
Neurosporene	tr	0.3	0.5	1.8	1.7	2.5
Lycopene	tr	10	38	52	48	36
γ-Carotene	tr	0.4	0.6	0.9	0.4	tr
β-Carotene	tr	0.9	2.0	1.1	0.6	tr

* Trace (< 0.1 μg/g)

Table 3 shows the results of a typical regeneration experiment. A 5-fold increase in lycopene concentration occurs during the first hour of resuspension, and this is accompanied by lesser increases in the levels of phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene), ζ-carotene (7,8,7′,8′-tetrahydro-ψ,ψ-carotene) and neurosporene (7,8-dihydro-ψ,ψ-carotene). While the level of 'total phytoene' shows very little change, there are

significant changes in the level of the all-*trans* isomer. These, however, represent an increase in concentration over the first hour of resuspension; although they do not show a precursor-product relationship with the levels of lycopene, they do demonstrate metabolic activity on the part of all-*trans* phytoene. The fact that the changes in all-*trans* phytoene concentration parallel those of other polyenes may indicate that this isomer is an intermediate between 15-*cis* phytoene and the more desaturated carotenes. A similar situation obtains in the purple non-sulphur photosynthetic bacterium, *Rhodospirillum rubrum*; recent studies[36] show an increase in the concentration of all-*trans* phytoene on the removal of DPA inhibition although, in this case, there is a detectable decrease in the level of total phytoene. The sudden changes (*Table 3*) in the polyene levels which occur between the harvesting and washing of the inhibited fungal culture and its resuspension in buffer, among which there is an apparent decrease in the all-*trans* phytoene concentration, would also support the view that it is a precursor of phytofluene.

Although these observations indicate metabolic activity on the part of the all-*trans* component of the accumulated phytoene, they do not demonstrate a net conversion of phytoene into its expected dehydrogenation products. They therefore present the same problems of interpretation that faced Goodwin when he showed that although the removal of DPA from an inhibited culture of *P. blakesleeanus* resulted in β-carotene formation, there was no corresponding reduction in the level of phytoene[15]. One of the possible conclusions which could be drawn from this observation is that the acyclic phytoene is not on the pathway of cyclic carotene formation[37]; such an explanation is obviously not applicable to the formation of the acyclic lycopene in the *carR21*(−) mutant and is therefore probably no more true for wild type *P. blakesleeanus*. Among further explanations that have been offered, one is particularly attractive in the light of recent work. It has been suggested that the accumulated phytoene may exist in the form of fat droplets[38] or has otherwise left a metabolically essential combination; the recent observation by Lee *et al.*[39] that the incubation of [^{14}C]GGPP with a cell-free system from the *car-10*(−) mutant of *P. blakesleeanus* results in the formation, not only of phytoene, but also of a phytoene–protein complex, which may be the metabolically active form of the polyene, lends considerable support to this view.

CONVERSION OF PHYTOENE INTO LYCOPENE

In order to demonstrate directly the conversion of phytoene into other carotenoids, it is necessary to use a cell-free system and to be able to solubilize the lipid-soluble [^{14}C]phytoene substrate in order to present it to the enzyme. Although the conversion of phytoene into phytofluene, lycopene and cyclic carotenes has been demonstrated in this way in higher plant systems[40,41], there are no similar reports for fungal systems.

In our studies with the crude enzyme system from the *carR21*(−) mutant of *P. blakesleeanus*, we have been able to demonstrate the enzymic conversion of [^{14}C]phytoene into other carotenes, with 6 and 2 per cent respectively of the added radioactivity recoverable on reisolation of added carrier phytofluene and lycopene[35]. The [^{14}C]phytoene was prepared using the

car-10(−) mutant and was added to the incubations in Tween 80. Although we have not yet attempted the demonstration of cofactor requirements for the dehydrogenation reactions, the anaerobic incubations were carried out in the dark (3 hours, 24°C) in the presence of 1 μmole/ml each of NAD^+, $NADP^+$, NADH, NADPH and FAD, together with 6, 4 and 20 μmoles/ml respectively of Mn^{2+}, Mg^{2+} and GSH. It has also been possible to demonstrate, using imidazole, phosphate, borate and Tris buffers, that the conversion of phytoene into lycopene is maximal at pH 8[36]. This is in contrast to the incorporation of [2-^{14}C]MVA into the carotenes by systems from this and other mutants; the latter incorporation is maximal at pH 8, but the use of imidazole buffer reveals high activity at pH 7. Investigations of this anomaly are in progress, but it is worth noting that mevalonic kinase from French bean leaves shows dual optima at pH 5.5 and 7.5[42,43].

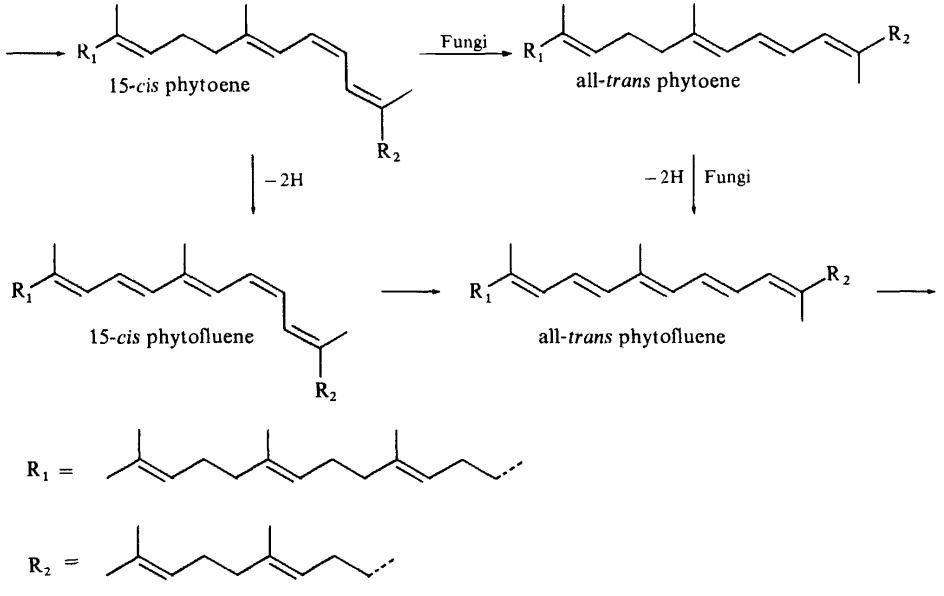

Figure 1. Possible pathways for the metabolism of 15-*cis* phytoene

The solubilizing agent, Tween 80, is not entirely passive in carotenogenic systems. A series of experiments, in which Tween 80, bovine serum albumin and ethylene glycol were individually assessed as possible solubilizing agents, showed that Tween 80 (2.5 mg/ml) reduces the incorporation of [2-^{14}C]MVA into the unsaponifiable fraction, the most marked effect being on carotene formation. At similar concentrations of Tween 80, however, the enzyme system is still capable of a significant conversion of phytoene into lycopene. The solubilizing agent appears to be exerting its effect, therefore, on the phytoene synthesizing system. A similar inhibition by Tween has been observed for some of the enzymes concerned in sterol ester formation[44] and

sphingosine biosynthesis[45], and with phosphatidic acid phosphatase[46] and glyceride transferase[47].

The relatively poor incorporations of phytoene into lycopene achieved by this cell-free system must be very largely due to the inability to present the substrate in the most satisfactory form to the enzyme; in the natural state, the phytoene is probably bound to a protein in some way[39]. Attempts to dilute out, by adding phytoene, the incorporation of [2-^{14}C]MVA into β-carotene by a crude enzyme system from the *mad-107*(−) mutant have yielded some interesting results[36]. While the addition of total natural phytoene (25 μg/ml; 3 per cent all-*trans*) results in a marked diminution in incorporation into β-carotene (*Table 6*), the addition of pure 15-*cis* phytoene has less effect. This may indicate that the all-*trans* isomer of phytoene is the true substrate of the dehydrogenation reaction or that it is exerting a powerful feedback effect.

It is too early to draw a firm conclusion on the way in which 15-*cis* phytoene is converted into the more unsaturated all-*trans* carotenoids, but all our results are consistent with the pathway shown in *Figure 1*. In this pathway, 15-*cis* phytoene is isomerized to the all-*trans* isomer which is then dehydrogenated to all-*trans* phytofluene; the alternative route, through *cis* phytofluene[41], is probably not as important in fungi as it appears to be in higher plant systems.

INTERMEDIATES IN THE DEHYDROGENATION OF PHYTOENE TO LYCOPENE

The Porter–Lincoln sequence, which represents the stepwise dehydrogenation of the phytoene molecule by the removal of two hydrogens alternately from either side of the central chromophore, results in the conversion of phytoene successively into phytofluene, the symmetrical ζ-carotene, neurosporene and lycopene[30,48]. The isolation from *Rhodospirillum rubrum* of the conjugated heptaene, 7,8,11,12-tetrahydrolycopene[49], and the absence of the isomeric ζ-carotene (7,8,7′,8′-tetrahydrolycopene), led to the concept of a different dehydrogenation sequence operating in photosynthetic bacteria[50]. In this sequence, the phytoene molecule is dehydrogenated twice on one side of the chromophore and then twice on the other, so that the intermediates between phytoene and lycopene are phytofluene, 7,8,11,12-tetrahydrolycopene and neurosporene. In at least one photosynthetic bacterium[51], and in *Flavobacterium dehydrogenans*[52], both pathways must operate, since both the isomeric conjugated heptaenes are detectable.

The recognition of the existence of different isomeric heptaenes stemmed from the initial observation of differences in the absorption spectra of samples of 'ζ-carotene' isolated from different sources. While ζ-carotene from higher plant tissues has absorption maxima in light petroleum at 378, 400 and 425 nm[50], the heptaene from *R. rubrum* has a spectrum showing peaks at 374, 394.5 and 418.5 nm in the same solvent[50]. It has been observed repeatedly that the absorption spectrum of the heptaene (all-*trans*[53]) isolated from *Neurospora crassa* has its maxima in light petroleum or in hexane at 375, 396–397 and 420–421 nm[14,54,55], that is, at values between those of the

two isomeric heptaenes; indeed, this spectral difference between the authentic ζ-carotene and the *N. crassa* pigment led Haxo to coin the name 'θ-carotene' for the latter pigment[54]. Other fungi, such as the *carR21*(−) and *mad-107*(−) mutants of *P. blakesleeanus* (but apparently not a wild type strain[50]), when cultured in the presence of DPA, also yield a conjugated heptaene with these spectral characteristics. On the basis of their absorption spectra and their mass spectra, which show $M^+ = 540$ and have ions at *m/e* 335 (M − 205), it is clear that samples of 'θ-carotene' from these organisms are mixtures of ζ-carotene and 7,8,11,12-tetrahydrolycopene, and that alternative pathways of phytoene dehydrogenation operate in these fungi (*Figure 2*).

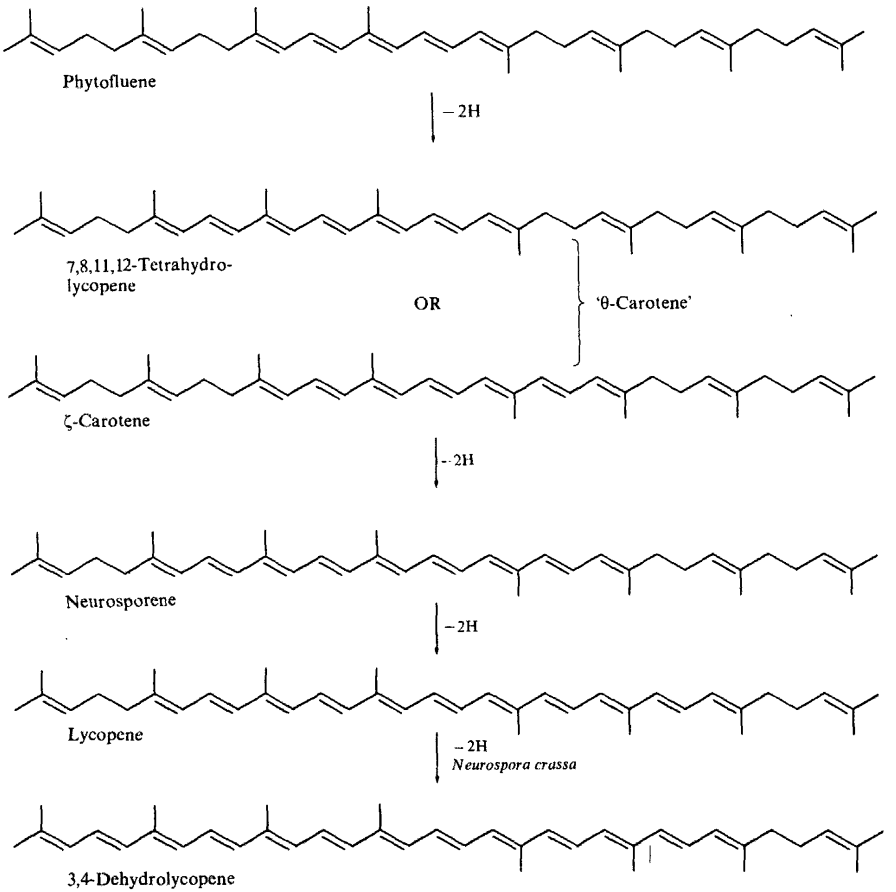

Figure 2. Alternative pathways for the dehydrogenation of phytofluene in fungi

The existence in cultures of *N. crassa* of 3,4-dehydrolycopene (3,4-didehydro-ψ,ψ-carotene)[55] raises an interesting consideration. If the dehydrogenating enzymes of this fungus are able to attack either side of the phytofluene chromophore, and so produce isomeric heptaenes, it might be anticipated that an enzyme capable of converting lycopene into its 3,4-dehydro derivative might also convert 7,8,11,12-tetrahydrolycopene into an 'isomeric neurosporene', i.e. 3,4-dehydro-7′,8′,11′,12′-tetrahydrolycopene (3,4-didehydro-7′,8′,11′,12′-tetrahydro-ψ,ψ-carotene). Studies of samples of the neurosporene fraction from *N. crassa* have failed to show the presence of this isomer; the mass spectrum of *N. crassa* neurosporene is normal with no ion at *m/e* 333 (M − 205). Thus a higher substrate specificity is apparent for the removal of hydrogens from carbons 3 and 4 than is the case for certain other dehydrogenations; the enzyme which converts lycopene into 3,4-dehydrolycopene is probably different from that (or, as is more likely, those) responsible for other dehydrogenations in *N. crassa*.

CAROTENE CYCLIZATION

Experiments with (4*R*)-[2-^{14}C-4-^{3}H$_1$]MVA, using *P. blakesleeanus*, have yielded a generally accepted mechanism of β-ring cyclization[19]. The experimental results indicate a loss of tritium from carbons 6 and 6′ of β-carotene and are therefore consistent with a mechanism in which protonic attack on a ψ-end group yields a formal carbonium ion which is stabilized by the loss of a proton from C-6 to give a β-ring (*Figure 3*).

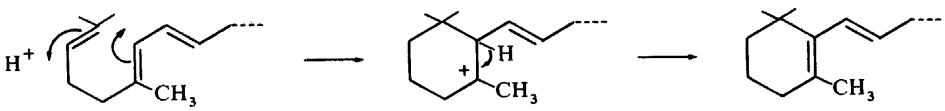

Figure 3. Mechanism of β-ring formation[19]

In spite of this, the current situation regarding the actual pathway of carotene cyclization in the fungi is no less confused than that for cyclization in other carotenogenic organisms. Experiments on fungi have produced evidence both for the cyclization of neurosporene to yield β-zeacarotene, which is then converted into β-carotene via γ-carotene (β,ψ-carotene), and for the direct cyclization of lycopene to γ-carotene and thence to β-carotene. Much of the evidence quoted in favour of neurosporene cyclization is circumstantial. The very existence of β-zeacarotene in fungi, both under conditions of DPA inhibition in *Rhodotorula*[56] and *P. blakesleeanus*[18] and in normal cultures of *N. crassa* (*Table 12*), means that these organisms have enzymes that will cyclize neurosporene. The decrease in β-zeacarotene concentration and the concomitant rise in the β-carotene level on removing the inhibitor from DPA cultures of *P. blakesleeanus*[17] is stronger evidence,

but is still indirect. Other evidence, more against lycopene as the cyclization substrate than specifically in favour of neurosporene, is that arising from experiments on the incorporation of [2-^{14}C]MVA into carotenes by *Rhizophlyctis rosea*, which forms only lycopene during the early stages of growth, with γ-carotene appearing later[57]. Incubation of a culture with the radioactive substrate during the later 'γ-carotene phase' only resulted in the lycopene and γ-carotene eventually extracted having specific radioactivities in the ratio of 1 to 17. The converse experiment, in which the [2-^{14}C]MVA was present during the early 'lycopene phase' and was then washed out of the culture, resulted in the lycopene and γ-carotene isolated at the end of the 'γ-carotene phase' having specific activities in the ratio of 6 to 1[58,59]. This evidence would tend to rule out the cyclization of lycopene in the organism, although a compartmentalization effect, in which the lycopene formed in the early stages of culture is deposited in metabolically inert lipid droplets, would provide an alternative explanation.

Although the more convincing evidence is for the cyclization of lycopene, the direct evidence comes from work on higher plants[60–63], and even this does not eliminate the possibility of neurosporene cyclization. The evidence from fungi is mainly two-fold: firstly, treatment of mycelia of *Blakeslea trispora* or *P. blakesleeanus* with CPTA [2-(*p*-chlorophenylthio)triethylamine hydrochloride][64] or of *P. blakesleeanus* with nicotine[65] results in the inhibition of β-carotene synthesis and the accumulation of lycopene; secondly, the *carR21*(−) mutant of *P. blakesleeanus* accumulates lycopene, rather than the β-carotene of the wild type (*Table 1*). All of these effects, however, can also be explained on the basis of neurosporene cyclization being inhibited, and the resultant accumulation of neurosporene being relieved by its dehydrogenation to lycopene. The conversion of lycopene into β-carotene on the removal of nicotine from an inhibited culture (as in species of *Flavobacterium*[65] and *Mycobacterium*[66]) need not necessarily indicate any more than a capability for lycopene cyclization under the prevailing conditions.

There is no evidence so far that cyclization can occur before the neurosporene stage of the dehydrogenation sequence, but the probable occurrence of 7,8,11,12-tetrahydrolycopene in *N. crassa* and in mutants of *P. blakesleeanus*, organisms which are capable of carotene cyclization, raises the question of whether this carotene might cyclize (*Figure 4*). It has the structural characteristics of a cyclizable polyene in that, as in neurosporene but not in ζ-carotene, dehydrogenation has proceeded as far as carbons 7 (or 7′) and 8 (or 8′) so that it could, theoretically, yield 7′,8′,11′,12′-tetrahydro-γ-carotene (7′,8′,11′,12′-tetrahydro-β,ψ-carotene). Such a carotenoid would have absorption maxima in light petroleum at about 359, 379 and 403 nm and should have adsorption characteristics similar to those of phytofluene. Large-scale extractions of *N. crassa* cultures, employing up to 500 g starting material, have failed to reveal any evidence of the existence of this carotenoid*. *N. crassa* cultures contain both 3,4-dehydrolycopene and torulene (3′,4′-didehydro-β,ψ-carotene)[55]; it is conceivable that alternative pathways exist

* 7′, 8′, 11′, 12′-Tetrahydro-γ-carotene has since been isolated from DPA-inhibited cultures of the *mad*-107(−) mutant of *P. blakesleeanus*[35].

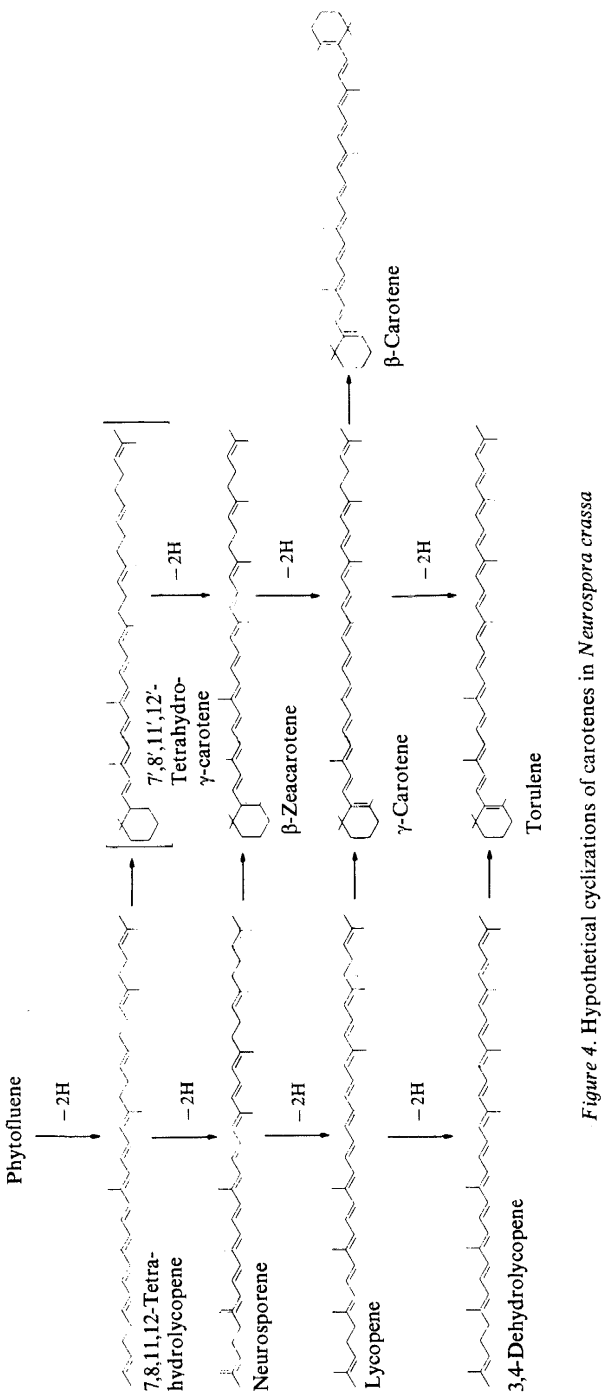

Figure 4. Hypothetical cyclizations of carotenes in *Neurospora crassa*

for torulene formation, either by the dehydrogenation of γ-carotene or by the cyclization of 3,4-dehydrolycopene. The theoretical possibilities for carotene cyclization in *N. crassa* are summarized in *Figure 4*.

EFFECT OF DIPHENYLAMINE ON CYCLIZATION

When the *carR21*(−) mutant of *P. blakesleeanus* is cultured in the presence of DPA, the relative levels of the carotenes vary with the DPA concentration[67]. The details of one such experiment are shown in *Figure 5*, while the data from a number of experiments are summarized in *Table 4*.

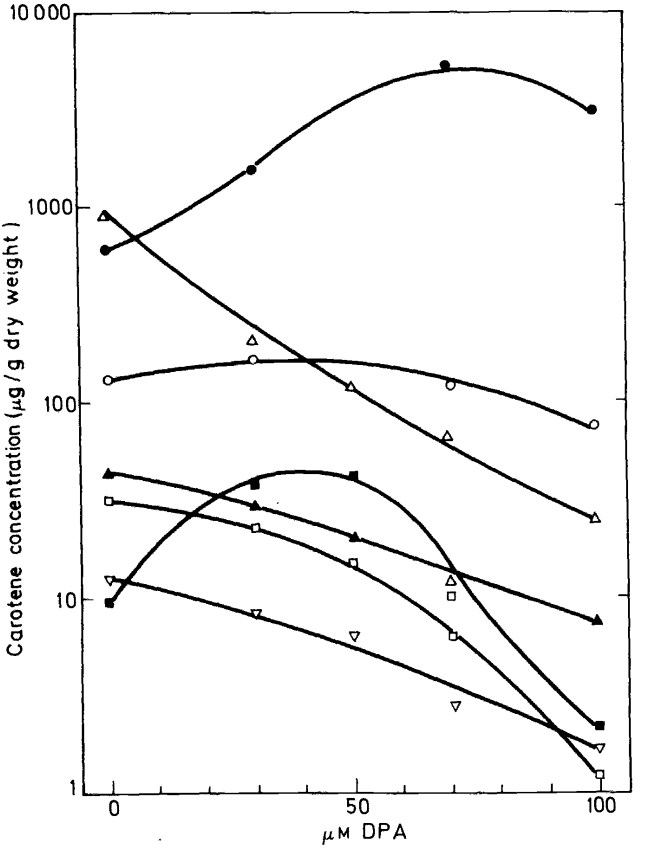

Figure 5. Effect of DPA concentration on carotene levels in the *carR21*(−) mutant of *P. blakesleeanus*

● Phytoene ○ Phytofluene ▲ ζ-Carotene
■ Neurosporene △ Lycopene ▽ γ-Carotene
□ β-Carotene

Phytoene accumulates at low levels of DPA but, at DPA concentrations above 60 μM, the level of phytoene, like the levels of all the other carotenes and the mycelial growth, is markedly reduced by the toxicity of the inhibitor. At concentrations of DPA between zero and 50 μM, the increase in phytoene

Table 4. Percentage changes in the levels of individual carotenes in *carR21*(−) mycelia caused by increasing the DPA concentration from zero to 50 μM

Carotene	Experiment 1	Experiment 2	Experiment 3
Phytoene	+207	+400	+630
Phytofluene	+50	+7	+100
ζ-Carotene	0	−70	+52
Neurosporene	+68	+91	+600
Lycopene	−87	−80	−83
γ-Carotene	−65	−45	+64
β-Carotene	−80	−69	−52

content with increasing DPA concentration is mirrored by a decrease in lycopene levels. Intermediates in the phytoene dehydrogenation sequence are also affected; while the level of phytofluene always shows a slight increase with DPA concentration, that of ζ-carotene, on average, stays fairly constant. On kinetic grounds, it would be expected that neurosporene, intermediate between ζ-carotene and lycopene, should show a correspondingly intermediate effect, falling in concentration as the DPA concentration increases, but less markedly than lycopene. Neurosporene, however, shows the opposite effect, always increasing in concentration. That this marked increase in concentration is always mirrored by a sharp decrease in the already low level of β-carotene, with γ-carotene showing an intermediate response, implies that neurosporene represents a branch point for cyclization.

DEMONSTRATION OF ALTERNATIVE ROUTES OF CAROTENE CYCLIZATION

In order to carry out a direct demonstration of the pathway of carotene cyclization, two different approaches have been used[36]. The first utilizes an enzyme system, from the *mad-107*(−) mutant of *P. blakesleeanus*, which is capable of converting [2-^{14}C]MVA into β-carotene and to investigate the effects on this incorporation of adding the various postulated intermediates of cyclization, namely neurosporene, β-zeacarotene, lycopene and γ-carotene. The enzyme system is normally capable of a 40–60 per cent incorporation of [2-^{14}C]MVA into the unsaponifiable fraction, with some 8–12 per cent of the added radioactivity appearing in the β-carotene, but these incorporations are reduced by the use of Tween 80 as a carrier for the added carotenes (see above) and are also extremely susceptible to variations in the age of the culture on harvesting (see later). In every incubation using this enzyme system, the β-carotene was recovered after carrier addition and was purified by one column and two thin-layer chromatographic systems. Its radiochemical purity was confirmed in a sufficient number of experiments by two recrystallizations. It has not been possible, so far, to demonstrate any cofactor requirement for β-carotene biosynthesis, apart from the ATP, Mg^{2+}, Mn^{2+} and GSH required for MVA utilization and for maintaining the stability of the *P. blakesleeanus* enzyme systems. All incubations, however, were supplemented with nicotinamide coenzymes and FAD as a routine procedure, and were carried out anaerobically in the dark at 24°C for 3 hours (maximum incorporation is achieved after 1.5 hours).

Initial experiments with added γ-carotene, which would be expected to dilute out the incorporation of [2-^{14}C]MVA into β-carotene irrespective of the precise nature of the pathway, were carried out in order to determine the optimum concentration of carotene additions. The recoveries of radioactivity in both β- and γ-carotenes were assessed and, as a result of these experiments (*Table 5*), subsequent studies used 26 μg (0.05 μmole) carotene in 2.5 mg Tween 80 per ml of incubation mixture.

Table 5. Radioactivities of β-carotene and γ-carotene formed from [2-^{14}C]MVA by a crude enzyme system from the *mad-107*(−) mutant of *P. blakesleeanus*

Additions per ml incubation*	β-Carotene (dpm)	γ-Carotene (dpm)
2.5 mg Tween 80	1186	515
2.5 mg Tween 80 + 5 μg (0.01 μmoles) γ-carotene	1158	333
2.5 mg Tween 80 + 26 μg (0.05 μmoles) γ-carotene	114	1522
2.5 mg Tween 80 + 53 μg (0.10 μmoles) γ-carotene	0	1529

* The incubation mixture contained DL-[2-^{14}C]MVA, 97 nmoles, 2.22 × 10^6 dpm; ATP, 10 μmoles; GSH, 20 μmoles; $MnCl_2$, 6 μmoles; $MgCl_2$, 4 μmoles; NAD^+, $NADP^+$, NADH, NADPH and FAD, 1 μmole each; Tris-HCl buffer pH 8.0, 200 μmoles and enzyme protein, 11.9 mg in final volume of 1 ml. Incubations were anaerobic in the dark for 3 hours at 24°C

The results of a complete study are shown in *Table 6*. The only carotenoid that did not dilute out the incorporation of radioactivity from [2-^{14}C]MVA into β-carotene was spirilloxanthin (1,1′-dimethoxy-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene) which, as it is not a fungal carotenoid[55], had been included merely as a control. The values are uncorrected with respect to the total incorporation into the unsaponifiable fraction, which varies from one incubation to another, and are therefore not directly comparable. Perhaps a better comparison can be made from the ratios of the radioactivity recovered in each case on reisolating and purifying the diluting carotene and β-carotene (*Table 7*). These figures show that, as might be expected, γ-carotene and then neurosporene trap the highest proportions of radioactivity; this situation is consistent both with neurosporene and

Table 6. Effect of adding unlabelled carotenoids on the incorporation of [2-^{14}C]MVA into β-carotene by a crude enzyme system from the *mad-107*(−) mutant of *P. blakesleeanus*

Addition	Radioactivity (dpm)* in recovered carrier carotenoids					
	β-Car.	γ-Car.	Neur.	Lyc.	β-Zea.	Spx.
Tween 80 only	506	—†	—	—	—	—
Tween 80 + γ-carotene	26	902	—	—	—	—
Tween 80 + neurosporene	464	—	2378	—	—	—
Tween 80 + lycopene	67	—	—	142	—	—
Tween 80 + β-zeacarotene	325	—	—	—	240	—
Tween 80 + β-carotene	175	—	477	492	—	—
Tween 80 + total phytoene	28	—	—	—	—	—
Tween 80 + 15-*cis* phytoene	381	—	—	—	—	—
Tween 80 + spirilloxanthin	546	—	—	—	—	6

Incubation mixture and conditions as in *Table 5* except that enzyme protein concentration was 13.4 mg/ml. Each carotenoid (0.05 μmole) was added with 2.5 mg Tween 80 per 1 ml incubation

* Values of dpm are not corrected with respect to total incorporation into unsaponifiable fraction

† Radioactivity not determined

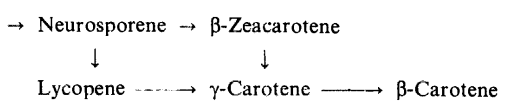

Figure 6. Conversion of neurosporene into β-carotene by *P. blakesleeanus*

Table 7. Ratios of radioactivities recovered in diluent carotenoid and in β-carotene after incubation of [2-^{14}C]MVA with a crude enzyme system from the *mad-107*(−) mutant of *P. blakesleeanus*

Carotenoid added to incubation mixture (i.e. diluent)	dpm in diluent : dpm in β-carotene
γ-Carotene	34.7 : 1
Neurosporene	5.12 : 1
Lycopene	2.12 : 1
β-Zeacarotene	0.74 : 1
Spirilloxanthin	0.01 : 1

Ratios calculated from radioactivities shown in *Table 6*

γ-carotene being on the pathway to β-carotene, irrespective of whether β-zeacarotene or lycopene is intermediate between them, and with γ-carotene being the immediate precursor of β-carotene. The ratios obtained with lycopene and with β-zeacarotene are both lower than that for neurosporene; this can only mean that neither β-zeacarotene nor lycopene, individually, is an obligatory intermediate in β-carotene formation and that alternative routes, through either lycopene or β-zeacarotene, actually operate in *P. blakesleeanus* (*Figure 6*). From this experiment at least, it may be concluded that the route through lycopene is, quantitatively, the more important.

INCORPORATION OF LABELLED CAROTENES INTO β-CAROTENE

The second approach to determining which of the alternative cyclization pathways operates in *P. blakesleeanus* is by investigating the direct incorporation of labelled carotene substrates into β-carotene. Three such substrates are currently available, namely lycopene, γ-carotene and neurosporene. [^{14}C]Lycopene has been prepared using the lycopene-accumulating *carR21*(−) mutant of *P. blakesleeanus*, either by adding [^{14}C]acetate to growing cultures or by incorporating the radioactivity from [2-^{14}C]MVA with a crude enzyme system. [^{14}C]γ-Carotene can also be prepared by the latter method from the *carR21*(−) mutant, but in low yield; better yields, but with lower specific activity, may be obtained by culturing *N. crassa* in the presence of a suitable ^{14}C-labelled substrate. High specific activity [^{14}C]neurosporene is obtained from a bacterial system. It has recently been shown in other studies[68] that nicotine, in addition to inhibiting carotene cyclization in bacteria and fungi, will also block the 1,2-hydration reaction which acyclic carotenes undergo in purple non-sulphur bacteria. Anaerobic cultures of *Rhodopseudomonas spheroides* normally contain spheroidene (1-methoxy-3,4-didehydro-1,2,7′,8′-tetrahydro-ψ,ψ-carotene) as the major

carotenoid; this is formed by a pathway which involves the hydration of neurosporene to chloroxanthin (1,2,7′,8′-tetrahydro-ψ,ψ-caroten-1-ol), methylation of chloroxanthin to yield 3,4-dihydrospheroidene (1-methoxy-1,2,7′,8′-tetrahydro-ψ,ψ-carotene) and, finally, dehydrogenation to spheroidene[68,69]. Thus *Rps. spheroides*, when cultured anaerobically in the presence of nicotine (7 mM), accumulates neurosporene as some 50–60 per cent of its total carotenoid, and the addition of [2,3-^{14}C]succinate to such cultures results in the formation of [^{14}C]neurosporene; specific radioactivities of up to 1.2×10^7 dpm/mg have been achieved in this way.

As was noted above, although the presence of Tween 80 substantially decreases the incorporation of [2-^{14}C]MVA into unsaponifiable components by enzyme systems from *P. blakesleeanus*, the direct incorporations of carotenes (e.g. of [^{14}C]phytoene into lycopene) are apparently less affected. The enzyme system from the *mad-107*(−) mutant has been incubated, in a series of experiments carried out under standard conditions, with the three carotene substrates, [^{14}C]lycopene, [^{14}C]γ-carotene and [^{14}C]neurosporene. In every case, radioactivity has been recovered on the addition and reisolation of carrier β-carotene, which has been purified to a constant specific activity. It appears from the results of this series of experiments (*Table 8*) that the percentage incorporation of a given carotene substrate into β-carotene depends on the concentration of substrate used; for both [^{14}C]lycopene and [^{14}C]neurosporene, the percentage incorporations at a substrate concentration of about 25 μg/ml are of the same order, but increase as the amount of substrate decreases. This effect is probably due to low concentrations of carotene giving a better percentage solubilization, so that high incorporations can only be achieved when substrates of very high specific activities are used. Under such conditions, a 6.9 per cent incorporation of [^{14}C]neurosporene into β-carotene was demonstrated, but this does not necessarily imply that neurosporene is a more efficient precursor of β-carotene than is lycopene; at equivalent substrate concentrations they have comparable efficiencies. Using the same argument, however, it is clear that γ-carotene gives the best incorporation, as would be expected from a substrate which is the immediate biosynthetic precursor of the product.

In order to assess whether the neurosporene/β-zeacarotene route or the lycopene/γ-carotene pathway is operating in the enzyme system, it is necessary either to compare [^{14}C]β-zeacarotene and [^{14}C]lycopene as substrates or to dilute out the incorporation of [^{14}C]neurosporene into β-carotene by the separate additions of unlabelled β-zeacarotene and lycopene. Since labelled β-zeacarotene is not readily available, the latter method has been used. In a series of incubations with the enzyme from the *mad-107*(−) mutant, [^{14}C]neurosporene was incubated alone, with unlabelled β-zeacarotene and with unlabelled lycopene. Carrier β-carotene was added in each case at the end of the incubation period and the recovered samples of β-carotene and the added carotenes were radioassayed after appropriate purification. The results (*Table 9*) indicate that β-zeacarotene and lycopene have an approximately equal effect in diluting out the incorporation of [^{14}C]neurosporene into β-carotene, so that both cyclization pathways (*Figure 6*) appear to be of equal quantitative importance. Of course, the lower incorporations into β-carotene in the presence of added carotenes may be due to the substrate

Table 8. Incorporations of [^{14}C]lycopene, [^{14}C]γ-carotene and [^{14}C]neurosporene into β-carotene by a crude enzyme system from the *mad-107*(−) mutant of *P. blakesleeanus*

[^{14}C]Substrate	Substrate concentration		Specific activity of substrate	Enzyme concn.	Specific activity of β-carotene	Percentage incorporation into β-carotene
	μg/ml	nmoles/ml	(dpm/mg)	(mg/ml)	(dpm/mg)	
Lycopene	23.5	43.8	3.32×10^5	13.8	7778* 7789†	2.6
Lycopene	2.8	5.2	3.25×10^6	9.0	50000* 43000† 44000‡	3.6
γ-Carotene	4.6	8.6	2.70×10^6	8.3	37717* 36775†	5.4
Neurosporene	28.8	53.5	8.21×10^5	18.8	19064* 17021†	3.3
Neurosporene	23.3	43.3	9.54×10^5	9.3	29278* 27281†	2.4
Neurosporene	0.79	1.5	1.16×10^7	10.7	55167* 37059† 39853‡	6.9

The incubation mixtures each contained labelled carotene and enzyme as indicated above, together with Tween 80, 2.5 mg; GSH, 20 μmoles; $MnCl_2$, 6 μmoles; $MgCl_2$, 4 μmoles; NAD^+, $NADP^+$, NADH, NADPH and FAD, 1 μmole each and Tris-HCl buffer pH 8.0, 200 μmoles in a final volume of 1 ml. Incubations were anaerobic in darkness for 2 hours at 24°C. At the end of each incubation, carrier β-carotene was added and reisolated and purified by:

* chromatography on columns of alumina
† column chromatography and thin-layer chromatography
‡ column chromatography and chromatography on two thin-layer systems

Table 9. Effect of added carotenes on the incorporation of [^{14}C]neurosporene into β-carotene by an enzyme system from the *mad-107*(−) mutant of *P. blakesleeanus*

Substrate and addition	Radioactivity (dpm) recovered as:			
	β-Car.	Lyc.	β-Zea.	Neur.
[^{14}C]Neurosporene only	527	—*	—	13913
[^{14}C]Neurosporene and β-zeacarotene	94	—	243	—
[^{14}C]Neurosporene and lycopene	112	297	—	—

The incubation mixtures each contained [^{14}C]neurosporene, 22300 dpm and 44 nmoles; Tween 80, 2.5 mg; GSH, 20 μmoles; $MnCl_2$, 6 μmoles; $MgCl_2$, 4 μmoles; NAD^+, $NADP^+$, NADH, NADPH and FAD, 1 μmole each; Tris-HCl buffer pH 8.0, 200 μmoles and enzyme protein, 12.95 mg in a final volume of 1 ml. β-Zeacarotene and lycopene were added to give final concentrations of 121 and 111 nmoles/ml respectively. Incubations were anaerobic in darkness for 2 hours at 24°C

* Radioactivity not determined

having a lower solubility at the higher total carotene concentration, but the trapping of equal amounts of radioactivity by the β-zeacarotene and the lycopene again leads to the conclusion that the two pathways are of equal significance. The labelling of the two added carotenes also demonstrates the *in vitro* conversion of neurosporene into both lycopene and β-zeacarotene.

It is impossible at this stage to make any assessment of whether the cyclization of neurosporene and that of lycopene are carried out by different specific enzymes or by the same, less specific, enzyme. Possibilities for alternate pathways exist at a number of points in the general pathway of carotenoid biosynthesis, at the phytoene isomerization and dehydrogenation

stage (*Figure 1*), for the dehydrogenation of phytofluene to neurosporene (*Figure 2*), at the cyclization stage (*Figure 4*) and, in photosynthetic bacteria, in the formation of methoxy carotenoids[69]. A final answer on whether these all represent examples of low-substrate specificity must await the isolation and study of the individual enzymes concerned.

EFFECT OF NICOTINE ON CYCLIZATION

The *mad-107*(−) mutant of *P. blakesleeanus*, like other carotenogenic organisms[65, 66], is sensitive to the presence of nicotine in the culture medium. The formation of β-carotene responds to nicotine over a wide range of concentrations up to 10 mM, when both growth and pigment synthesis are reduced to zero. At lower concentrations of the inhibitor (2.5 and 5 mM), the inhibitory effect of nicotine is already apparent; at a 7 mM concentration, β-carotene formation is totally inhibited and lycopene accumulates (*Table 10*).

Table 10. Effect of nicotine on the concentrations of β-carotene and lycopene formed by mycelia of the *mad-107*(−) mutant of *P. blakesleeanus*

Nicotine concentration (mM)	Carotene concentrations (μg/g dry weight) β-Carotene	Lycopene
0	3780	0
1.0	3585	36
5.0	2120	1230
7.0	5	2660

Trace amounts of γ-carotene (1–5 μg/g) were not determined quantitatively

Table 11. Effect on carotene levels of removing nicotine from a culture of the *mad-107*(−) mutant of *P. blakesleeanus* which has been grown in the presence of nicotine (7 mM)

Time after resuspension (hours)	Carotene concentrations (μg/g dry weight) β-Carotene	γ-Carotene	Lycopene
0	41.2	9.7	1277
1.0	76.4	1.7	1061
2.5	471	13.4	1183
10.0	668	13.8	1551

Removal of nicotine from such a culture and resuspension in buffer, however, did not result in the expected change in colour from red to yellow. Analysis of the carotenes at various times after resuspension (*Table 11*) confirmed that the lycopene concentration did not decrease very markedly although there was a continuous increase in the level of β-carotene, indicating that the inhibitory effect of the nicotine had been removed. The small amount of γ-carotene formed under conditions of nicotine inhibition decreased in the first hour after resuspension and then rose and stabilized over the next 2 hours.

A possible explanation for the failure to demonstrate the expected precursor-product relationship between lycopene and β-carotene might be

that the large amount of lycopene accumulated under conditions of nicotine inhibition had been lost from association with protein at its site of metabolism and had become metabolically inert on entering a lipid pool.

EFFECT OF LIGHT ON CAROTENE FORMATION

All the incubations of crude extracts from the mutants of *P. blakesleeanus* were carried out in the dark. Preliminary experiments had shown that the incorporation of [2-^{14}C]MVA into lycopene and other terpenoid components of the *carR21*(−) mutant were independent of illumination, so dark conditions were chosen as a routine procedure because of the photosensitivity of the carotenes.

In culturing the mutants of *P. blakesleeanus*, no absolute requirement for light, either for the formation of any individual carotene or for carotene biosynthesis as a whole, could be demonstrated although it is clear that cultures grown in the light have carotene concentrations some 20–50 per cent (depending on the mutant) higher than those maintained in the dark. The figures in *Table 1* for the carotene contents of the mutants cultured in light and in darkness refer to shake cultures; those grown in the dark were inoculated in darkness from spore suspensions prepared in the dark from dark-grown static cultures. It is apparent from visual observations that static cultures tend to show more response to light in terms of their growth and carotene content than do shake cultures.

In *N. crassa* also, light has a stimulatory effect on carotene formation, both in terms of the concentration of pigment and the type of carotene formed. The figures in *Table 12* indicate that while the total carotene content

Table 12. Effect of light on carotene levels in *Neurospora crassa*

Carotene	Concentration (μg/100 g wet wt.)*		Percentage of total carotene		Percentage of total carotene excluding phytoene	
	Dark	Light	Dark	Light	Dark	Light
Phytoene	2370	4650	91.04	73.63	—	—
Phytofluene	74	189	2.84	2.99	31.75	11.35
θ-Carotene	47	364	1.80	5.76	20.17	21.86
Neurosporene	79	143	3.03	2.26	33.90	8.58
Lycopene	10	102	0.38	1.61	4.29	6.12
3,4-Dehydrolycopene	tr†	18	—	0.28	—	1.08
β-Zeacarotene	tr	91	—	1.44	—	5.46
γ-Carotene	12	533	0.46	8.44	5.15	32.01
Torulene	tr	tr‡	—	—	—	—
β-Carotene	11	225	0.42	3.56	4.72	13.51
Total acyclic carotenes	2580	5466	99.11	86.55	90.12	49.00
Total cyclic carotenes	23	849	0.88	13.44	9.87	50.99
Total carotene	2603	6315	99.99	99.99	99.99	99.99

* Yields of mycelium from 500 ml culture medium (static) were 110 g in dark and 116 g in light

† Trace (< 5 μg/100 g)

‡ Another strain of *N. crassa* gave more torulene (31.7 μg/100 g), but the overall pattern of carotene distribution was the same

increases only 2- or 3-fold, there is a 30-fold increase in the concentration of cyclic carotenes, although this increase is not so obvious on a percentage basis because of the large amounts of phytoene that occur in both light and dark cultures.

GROWTH AND CAROTENE FORMATION

Mention has already been made of the sensitivity on the part of the activity of the *P. blakesleeanus* enzymes to the time of harvesting the fungal culture. The reason for this became clear when a study was made of the growth characteristics of the cultures, including enzyme activities, on a time basis. The results of two such studies, on the red *carR21*(−) and the yellow *mad-107*(−) mutants, are presented in *Figures* 7 and 8 respectively.

After the inoculation of liquid-shake cultures with a standard spore suspension, and incubating in the light at 24°C, there was a lag period of 18–24 hours before growth commenced. Growth continued for a further 60 hours before

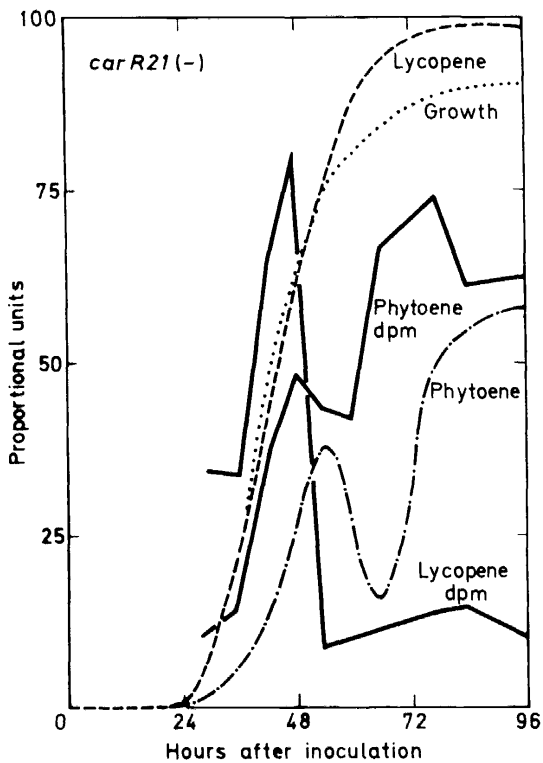

Figure 7. Growth and carotene formation in the *carR21*(−) mutant of *P. blakesleeanus*. Curves show: growth (100 ≡ 700 mg dry wt./100 ml); lycopene concentration (100 ≡ 1500 μg/g dry wt.); phytoene concentration (100 ≡ 1500 μg/g dry wt.); [2-^{14}C]MVA incorporation into lycopene (100 ≡ 250 dpm in lycopene/mg protein); [2-^{14}C]MVA incorporation into phytoene (100 ≡ 3000 dpm in phytoene/mg protein). Incubation conditions for enzyme assays were as in *Table 5*

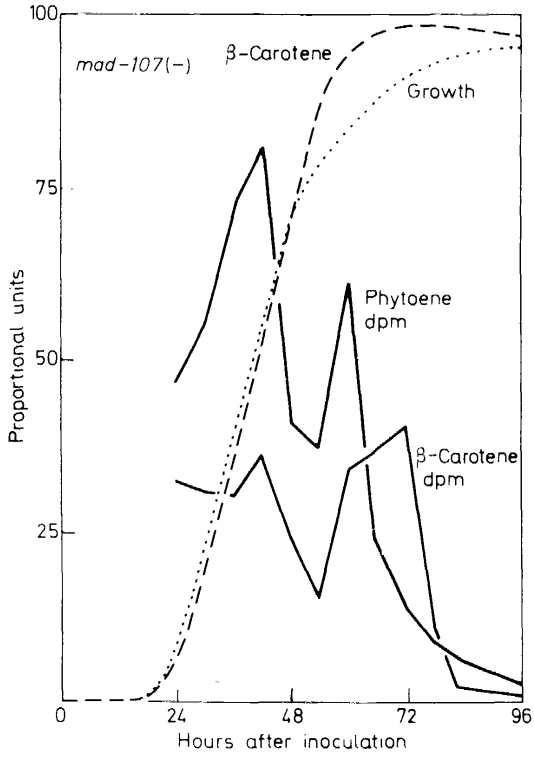

Figure 8. Growth and carotene formation in the *mad-107*(−) mutant of *P. blakesleeanus*. Curves show: growth (100 ≡ 750 mg dry wt./100 ml); β-carotene concentration (100 ≡ 2500 μg/g dry wt.); [2-^{14}C]MVA incorporation into β-carotene (100 ≡ 250 dpm in β-carotene/mg protein); [2-^{14}C]MVA incorporation into phytoene (100 ≡ 3000 dpm in phytoene/mg protein). Incubation conditions for enzyme assays were as in *Table 5*

the dry-weight yield levelled off. The increases in the lycopene content of the *carR21*(−) mutant and in the β-carotene content of the *mad-107*(−) cultures followed the growth curves, each carotene reaching a maximum some 72–84 hours after inoculation. While analyses of the *mad-107*(−) cultures revealed no phytoene, those of the *carR21*(−) samples showed that the concentrations of phytoene, phytofluene and ζ-carotene increased up to 54 hours, decreased, and then rose again to reach maximum values some 84 hours after inoculation.

Although phytoene was not detectable in the *mad-107*(−) cultures, the incubation of [2-^{14}C]MVA under the standard conditions with protein extracts from this mutant, and the subsequent addition of carrier phytoene, β-carotene and other terpenoids, resulted in radioactivity appearing in both of the reisolated carotenes. It was possible, using this technique, to show that the incorporation of [2-^{14}C]MVA into both phytoene and β-carotene (and into squalene) took place in two distinct phases, with the protein showing maximal enzyme activity at 42 and 60–72 hours after inoculation.

In the latter phase, the maximum incorporation into phytoene preceded that into β-carotene by some 12 hours.

In the case of the *carR21*(−) mutant, two synthetic phases were again apparent, although in this case radioactivity from [2-^{14}C]MVA was incorporated into lycopene with maximum efficiency only during the first phase (the highest specific activity of the enzyme was recorded at 42 hours). While phytoene was formed by the protein extract during both phases, the highest amounts were produced during the period 66–78 hours after inoculation, thus accounting for the accumulation of phytoene observed on analysis of the carotenes.

The incorporation of radioactivity by the *carR21*(−) mutant into phytoene rather than into lycopene in the latter stages of growth reveals what may be a feedback inhibition, mediated by a high concentration of lycopene, of the enzymes which dehydrogenate phytoene (*Figure 9*). This situation, apparently, does not occur in the β-carotene-accumulating mutant, *mad-107*(−); the formation of β-carotene from [2-^{14}C]MVA can occur right up to the stationary phase of growth, and high concentrations of β-carotene do not inhibit phytoene metabolism.

ASPECTS OF THE CONTROL OF CAROTENE BIOSYNTHESIS

As work on the *car-10*(−) mutant of *P. blakesleeanus* has confirmed, the channelling of terpenoid biosynthesis into sterols, squalene and carotenes depends on the availability of oxygen and NADPH. In the course of the investigations of carotene biosynthesis in the *mad-107*(−) mutant, however, a number of observations were made which pointed to the existence of a subtler series of control mechanisms, operating on the negative-feedback principle.

Table 13. Effect of terpenoid additions on the ratio of radioactivities incorporated into phytoene and squalene after incubation of [2-^{14}C]MVA with a crude enzyme system from the *mad-107*(−) mutant of *P. blakesleeanus*

Addition	dpm in phytoene : dpm in squalene
Tween 80 only	0.80 : 1
Tween 80 + squalene	0.80 : 1
Tween 80 + total phytoene	0.85 : 1
Tween 80 + 15-*cis* phytoene	0.79 : 1
Tween 80 + neurosporene	1.78 : 1
Tween 80 + lycopene	1.55 : 1
Tween 80 + β-zeacarotene	1.84 : 1
Tween 80 + γ-carotene	3.56 : 1
Tween 80 + β-carotene	0.91 : 1
Tween 80 + spirilloxanthin	0.81 : 1

Incubation conditions as in *Table 6*

Typical of these observations are those indicated in *Table 13*. These figures show that the addition of certain carotenes to a system forming β-carotene and other terpenoids from [2-^{14}C]MVA affects the relative

amounts of phytoene and squalene that are formed. The ratio of phytoene to squalene, which is normally 0.8 to 1 in control experiments, is unchanged by the addition of squalene, phytoene or spirilloxanthin (not a fungal carotenoid[55]), while the addition of neurosporene, β-zeacarotene, lycopene or γ-carotene increases the ratio by a factor of at least 2. The effect of β-carotene is less marked than those of the other coloured carotenes. These apparent increases in the proportion of phytoene formed may indicate the operation of a feedback mechanism, in which high concentrations of the carotenes inhibit the dehydrogenation of phytoene. The lower phytoene to squalene ratio in the case of β-carotene addition parallels the failure of this carotene to cause phytoene accumulation in the later stages of fungal growth.

The addition of β-carotene, however, does cause inhibition of the cyclization reactions for, as the results in *Table 6* show, β-carotene apparently inhibits, to some extent, its own formation and causes the accumulation of radioactivity in neurosporene and lycopene instead. The addition of either phytoene or squalene, while not affecting the ratio of incorporations, causes parallel reductions of the incorporations of [2-^{14}C]MVA into both squalene and phytoene[36]. This may be indicative of the existence of a further feedback-control mechanism in which accumulations of either phytoene or squalene can slow down the overall rate of terpenoid biosynthesis by inhibiting one

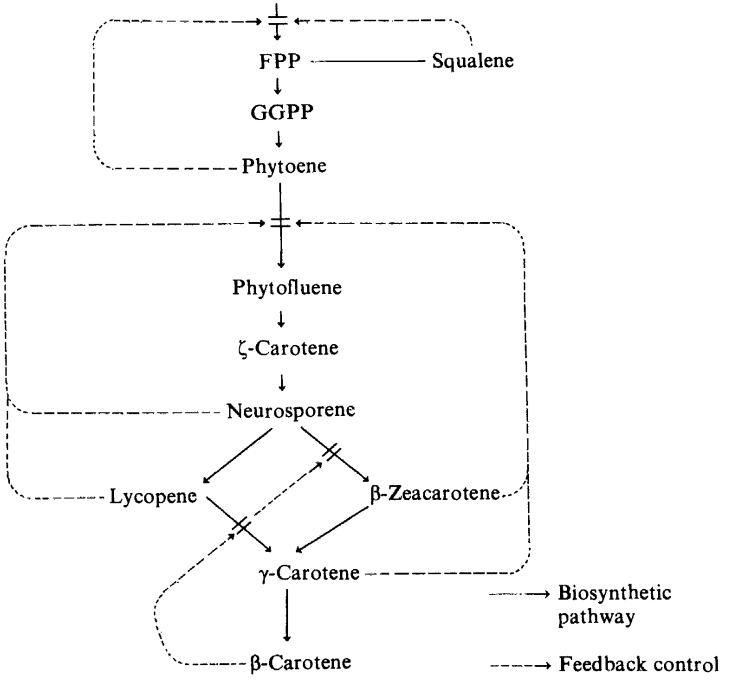

Figure 9. Postulated feedback control mechanisms for carotene biosynthesis in *P. blakesleeanus*

of the enzymes operating between MVA and FPP. These postulated negative-feedback mechanisms are indicated in *Figure 9*.

ANALYSIS OF THE ENZYME PROTEINS

Continuation of these studies on the biosynthesis of carotenes by cell-free systems must clearly involve attempts to purify the enzymes responsible for catalysing the key steps in the biosynthetic pathway. The protein compositions of the crude enzyme systems have been compared by isoelectric focusing on polyacrylamide gel (*Figure 10*). The most complex separation pattern was achieved using an extract from the wild type *P. blakesleeanus*, while the simplest combination of proteins is present in extracts of the phytoene-accumulating *car-10*(−) mutant. It is tempting to speculate that the protein bands absent from extracts of the white mutant may represent the enzymes which are responsible for converting phytoene into β-carotene.

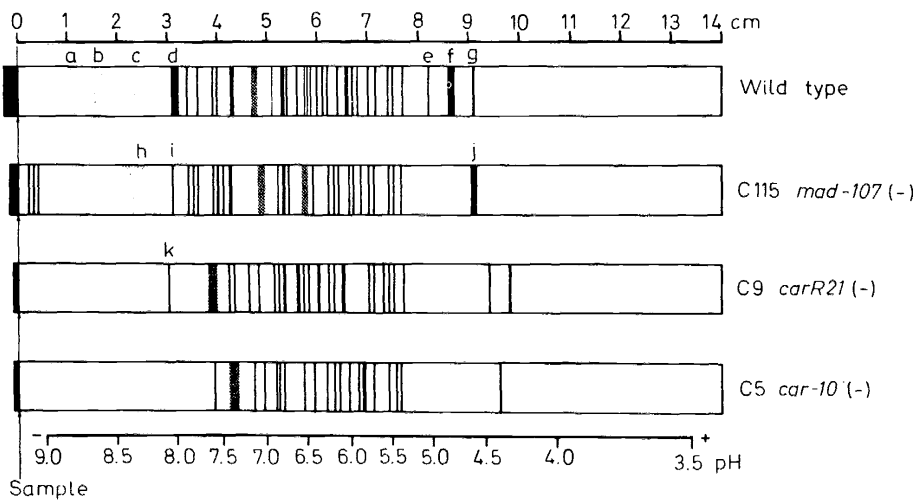

Figure 10. Polyacrylamide gel isoelectric focusing of active extracts from mutants of *P. blakesleeanus*. Sample, 350-400 μg protein in 25 μl buffer; gel, 5 per cent acrylamide and 1 per cent carrier ampholyte (LKB), pH 3 10; conditions, 1 mA per tube at 500 V (max.) for 3.5 hours at 4°C; stain, Amido Black

Not all the proteins, of course, necessarily have any connection with carotene biosynthesis, but it is worth noting that the corresponding bands (c and h) at pI 8.3 and those (g and j) at pI 4.6 of the wild type and the *mad-107*(−) mutant are absent from those strains which do not produce β-carotene as the main carotene, so that either band, or both, could represent the cyclization system. Quantitative comparisons show that the *mad-107*(−) band at pI 4.6 (j) is particularly intense, perhaps reflecting the ability of this mutant to form very large amounts of β-carotene. On the basis that they are absent from active extracts of the white *car-10*(−) mutant, it could be argued that

the bands at pI 8.0–8.1 (d, i and k) may represent the enzyme or enzymes which dehydrogenate phytoene to the coloured acyclic carotenes.

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